Epigenetic differences between male and female bovine blastocysts produced in vitro

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IN EURHEAN MAMMALS, EVIDENCE has emerged that clearly demonstrates differences in growth rates and metabolism between male and female embryos that appear before sexual differentiation of the gonads and, therefore, could not be explained by sex-related hormonal differences (6, 36). In preimplantation bovine embryos, total glucose metabolism is twice as high in male embryos as female embryos, and the activity of the pentose phosphate pathway is four times greater in female than in male blastocysts (54). Similar metabolic differences were found in human embryos at this stage (46). Differential metabolism and growth rates may be attributable to the unbalanced expression of X-linked genes between the sexes during certain stages of early preimplantation development, where both X chromosomes may be active (32, 40). We have shown that mRNA relative abundance of three X-linked genes is expressed at higher levels in female bovine embryos than in male embryos at the early blastocyst stage: two are important components of energetic metabolism, also involved in controlling the amount of oxygen radicals [glucose-6-phosphate dehydrogenase (G6PD) and hypoxanthine phosphoribosyltransferase (HPRT)] and the third, X-linked inhibitor of apoptosis protein (XIAP), is a mammalian protein that controls apoptosis through modulation of caspase activation and activity (19, 24). This differential expression has also been confirmed in other species (53, 58). The development of genomic procedures such as transgenesis and microarray analysis has allowed the discovery of nearly 600 differentially expressed genes between male and female mouse blastocysts (29). These results confirm differences previously reported in cattle (17).

Evidence from several species indicates that embryos produced in vitro that reach the blastocyst stage earliest are more likely to be males than females; examples include the mouse (55), cow (1, 17), human (44), pig (8), and sheep (4). Furthermore, the faster-developing blastocysts in in vitro culture systems are generally considered more viable and better able to survive cryopreservation or embryo transfer than those that develop more slowly (38). However, under suboptimal conditions, female embryos are more resistant than male embryos (43). This suggests that some early differences between male and female embryos are manifested under certain environmental conditions (18), and these early differences may be related to the control of the secondary sex ratio in mammals (i.e., differences in sex ratio observed at birth) (23). The mechanism(s) responsible for the observed phenotypic differences between male and female embryos in rate of development to the blastocyst stage is not clearly understood. Mitochondria, which play a central role in the provision of energy to embryo, may play a role in the differences in development between the male and female embryos (37), because enhanced rates of cell proliferation in the developing male embryo may be expected to require increased levels of cellular energy. Moreover it has been reported that mitochondria distribution at preimplantation stage may be an epigenetic factor developmentally relevant with respect to embryo competence (56). In addition, telomere elongation during embryogenesis is restricted to the preimplantation morula-blastocyst transition (49), and it is possible that the differences between sexes at early mammalian embryos have a telomerase-dependent genetic program that elongates telomeres to a defined length (49). Telomere length is also related to the epigenetic status of mammalian cells. It has been reported that telomere length regulates the epigenetic...
status (histone modifications and DNA methylation) of mammalian telomeres and subtelomeres (3). Also, during the pre-implantation period, there is a relationship between genetic and epigenetic reprogramming (13); for this reason one could expect that the changes observed in gene expression between male and female embryo may be a cause or consequence of changes in epigenetic events. These epigenetic events may have a long term sex-linked effect in the adult animal (12) or may be hereditary and lead to sex-specific transgenerational responses (42).

Because in vitro culture may be responsible for or, at the very least, exaggerate the sex differences observed in embryos, bovine in vitro culture represents an excellent model to analyze genetic and epigenetic differences between male and female embryos because the oocyte takes >7 days to develop to blastocyst stage; this long period should help to amplify these differences (20). The aim of this study was to examine sex-related differences in mtDNA content, telomere length, methylation of different regions of the genome, and mRNA transcription of genes related with cytosine methylation and histone methylation in bovine blastocysts produced in vitro.

MATERIALS AND METHODS

Semen preparation. Semen was collected from a Holstein Frisian bull of proven fertility and diluted immediately with Sexcess extender (Masterring, Verden, Germany) to a concentration of 1 × 10^8 sperm/ml. Spermatozoa were labeled with 15–25 µl of a 8.12 mM Hoechst 33342 solution (8.9 mM of 2-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2.5-bi-1H-benzimidazole in bi-distillated water) for 90 min at 34°C. Sperm sorting was performed according to the Beltsville Sperm Sorting Technology (25). Labeled sperm samples were filtered through a 51 µm cell strainer grid (Falcon Becton Dickinson, Franklin Lakes, NJ) and then supplemented with 1 µl food dye solution FD&C#40 (Warner Jekinson, St. Louis, MO). Sorting was performed with a high-speed flow cytometer (MoFlo SX, DakoCytomation, Fort Collins, CO), equipped with an argon UV-Laser (Coherent Laser, Inova I 90C-6; Dieburg, Germany), set to 200 mW output. Samples were sorted at an average event rate of 25,000 cells/s, giving a sorting rate of 3,300 cells/s. Spermatozoa were collected into 10 ml conical plastic tubes (Greiner, Nürtingen, Germany) prefilled with 500 µl TEST-yolk extender (25). Immediately after collection of 8 million spermatozoa, the sorted cells were centrifuged at 840 g for 20 min. The supernatant was discharged, and the pellet was resuspended with a TRIS-based cooling extender and cooled to 4°C within 2 h. Then, the final sperm concentration was set 20.6 × 10^6 sperm/ml with a TRIS-based freezing extender (28), and 3.3 × 10^6 spermatozoa were filled into 0.25 ml plastic straws (Minüt, Tiefenbach, Germany) (segment 1 with 160 µl sperm and segment 2 with 50 µl extender), sealed and frozen in liquid nitrogen. From each sorted frozen sample a purity analysis for the correct sex separation was performed using a flow cytometrical resort protocol and by curve fitting statistics [Gaus 7, (25)].

Blastocyst production. Immature cumulus oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of heifers and cows at slaughter. COCs were matured for 24 h in TCN-199 supplemented with 10% (vol/vol) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor at 39°C under an atmosphere of 5% CO₂ in air with maximum humidity. For in vitro fertilization (IVF), matured COCs were inseminated with frozen-thawed, Percoll-separated, flow-cytometrically sex-sorted (X-sorted; n = 1,057 or Y-sorted; n = 1,094) and unsorted (control; n = 157) bull sperm at a concentration of 1 × 10^6 spermatozoa/ml. On each day of IVF a small number of oocytes were inseminated with unsorted semen as a control to ensure procedures in the laboratory were optimal, hence the lower numbers. Gametes were co-incubated at 39°C under an atmosphere of 5% CO₂ in air with maximum humidity. At ~20 h postinsemination (hpi), presumptive zygotes were denuded and transferred to 25 µl culture droplets (1 embryo per µl) under mineral oil. Culture took place in synthetic oviduct fluid + 5% FCS. Plates were incubated for 7 days at 39°C under an atmosphere of 5% CO₂, 90% of N₂, and 5% O₂ with maximum humidity. Day 7 blastocysts from both experimental groups were snap-frozen in groups of 10 for analysis of mRNA relative abundance, mtDNA, and methylation status. Five replicates were performed, and embryos from several replicates were used in the genetic and epigenetic analyses.

Embryo sexing by PCR. A preliminary study was performed for verification of the sorting procedure. All blastocysts used were produced in vitro as described above. Day 7 blastocysts from both groups (X-sorted; n = 47, Y-sorted; n = 61) were first washed in PBS and then transferred into 5 mg/ml Pronase (Sigma P5147, Madrid, Spain) in PBS medium for 1 min to remove the zona pellucida and any attached spermatozoa. They were then washed three or four times in PBS and individually snap-frozen in liquid nitrogen in Eppendorf tubes and stored at −80°C until analysis. Samples were thawed at room temperature and centrifuged at 8,000 g for 1 min prior to being mixed with PCR reagents. Two sets of PCR primers were used to determine embryo sex: Y-chromosome-specific primers (BRY4a) and bovine-specific satellite sequence primers (Sat1) (33). Because of the number of repetitions of these sequences, this is one of the best systems to sex bovine embryos in a single PCR. The amplification reactions were conducted in a total volume of 25 µl containing 1× PCR buffer, 2 mM MgCl₂, 0.5 mM dNTPs, 1 unit of Taq DNA polymerase, 0.1 mM of the Sat1 primer, and 0.3 mM of the Bry4a primer. PCR was programmed for 35 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 20 s; in the first cycle denaturation was at 95°C for 3 min, and after the 35 cycles the reaction mixtures were kept at 72°C for 5 min. PCR product were analyzed on 2% agarose gel and ethidium bromide staining. The gel was visualized under ultraviolet illumination for the positive 300 bp band of Bry4a 1a and 216 bp of the satellite sequence. Samples that exhibited both bands were assigned as males, while the samples exhibiting only a satellite sequence band were assigned as female (Fig. 1). Quantification of mtDNA. To analyze mtDNA, 60 individual day 7 blastocysts and six groups of five blastocysts of each sex from three different experimental replicates were used. DNA was extracted from each sample and used directly for PCR analysis as described by Shitara et al. (50) and divided in three aliquots; each aliquot was used directly for sexing, mtDNA quantification (35), and measurement of telomere length by PCR. For mtDNA quantification, we used primers located in the COX1 gene (Table 1) that amplify a product of 190 bp (there is only one copy of the gene in the mitochondria genome). Quantification was performed by a real-time polymerase chain reaction (qRT-PCR) method. Briefly, a Rotorgene 2000 Real Time CyclerTM (Corbett Research, Sydney, Australia) and SYBR Green (Molecular Probes, Eurogene, OR) as a double-stranded DNA-specific fluorescent dye were used to determine the mtDNA copy number. The PCR reaction mixture (25 µl) contained 1× PCR buffer, 2 mM MgCl₂, 2 U Taq Express (MWGAG Biotech, Ebersberg, Germany), 100 µM of each dNTPs, and 0.2 µM of each primer. In addition, the double-stranded DNA dye, SYBR Green I, (1:3,000 of 10,000× stock solution) was included in each reaction. The PCR protocol included an initial step of 94°C (2 min), followed by 40 cycles of 94°C (15 s), 56°C (30 s) and 72°C (10 s). Fluorescent data were acquired at 83°C after the elongation step. The melting protocol consisted of a hold temperature at 40°C for 60 s and then heating from 50 to 94°C, holding at each temperature for 5 s while monitoring fluorescence. Product identity was confirmed by ethidium bromide-stained 2% agarose gel electrophoresis. The external standard was a COX1 PCR standard product that was purified and quantified by spectrophotometry, assuming that 1 ng of a 200 bp product contained 4.5 × 10⁹ molecules of double-stranded DNA. Several serial dilutions were then

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real-time quantitative PCR method previously described (7). DNA was extracted from each blastocyst as described by Shitara et al. (50) and divided in three aliquots; each aliquot was used directly for sexing, mtDNA quantification, and measurement of telomere length by PCR analysis. Quantification of telomere length by real-time PCR was performed according to the relative standard curve protocol, doing a minimum of three repetitions for each sample. The assay measures an average telomere length ratio (ATLR) by quantifying telomeric DNA with specially designed primer sequences and dividing that amount by the quantity of a single-copy gene (H2a.z). To serve as a reference for standard curve calculation, an individual sample of bovine somatic DNA was serially diluted over a 25-fold range for the telomere PCR and over 16-fold range for the H2a.z. The relative amount of the telomere PCR product was divided by the relative amount of the H2a.z, and the ratio of telomere/H2a.z was calculated. The average of these ratios was reported as the ATLR. The primers used for RT-PCR are listed in Table 1.

Bisulphite treatment, PCR, and restriction analysis of PCR products. To analyze methylation status, three groups of 10 embryos of each sex from three different experimental replicates were used. Embryos were washed in PBS, placed in 1.5 ml Eppendorf tubes, snap-frozen, and stored at −80°C until they were analyzed. As a control, the DNA of ~1 mm² of ovarian stroma was extracted with phenol-chloroform and diluted. The DNA was treated with sodium bisulphite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Bisulphite-modified DNA was used to amplify each sequence. The methylated status of a part of the satellite I region, a part of the satellite II region, a part of the 18S rRNA sequence, a part of the Alu-like short interspersed nuclear element (SINE) art2, and a part of the 18S rRNA sequence were amplified using nested or hemi-nested PCR (26, 27). The first PCR consisted of one cycle of 94°C for 2 min, 45°C for 30 s and 72°C for 20 s, followed by 4 cycles of 94°C for 1 min, 50°C for 30 s, and 72°C for 20 s, 35 cycles of 94°C for 15 s, 54°C for 30 s, and 72°C for 20 s, and a final step of 72°C for 8 min. The nested PCR was carried out using 1 µl of the product and consisted of a first step of 94°C for 2 min, 35 cycles of 94°C for 20 s, 54°C for 30 s, and 72°C for 20 s, and a final step of 72°C for 8 min. Primers are indicated in Table 2. Cytokeratin gene promoter sequence was amplified using a nested PCR consisting of one cycle of 94°C for 3 min, 55°C for 40 s, and 72°C for 20 s, followed by 4 cycles of 93°C for 1 min, 56°C for 30 s, and 72°C for 20 s, 25 cycles of 94°C for 15 s, 57°C for 30 s, and 72°C for 20 s, and a final step of 72°C for 8 min. From the PCR products, 15 µl were digested with 1 UI of AciI restriction enzyme (New England Biolabs, Hitchin, Herts, UK) overnight at 37 °C for the promoter sequence of cytokeratin gene, satellite I, and divided in three aliquots; each aliquot was used directly for sexing.

Table 1. Details of primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>Fragment Size, bp</th>
<th>Annealing Temperature, °C</th>
<th>Gene Bank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H2a.z</td>
<td>AGGAGGACTGAGCTAGGAGCTTG</td>
<td>208</td>
<td>59</td>
<td>NM_174809</td>
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<tr>
<td>COX1</td>
<td>GCCACCAGCCAGATTTTGAACCTTG</td>
<td>190</td>
<td>59</td>
<td>KY676873</td>
</tr>
<tr>
<td>Dmnt1</td>
<td>GCCATGAGCCTACGATGACCTTTT</td>
<td>311</td>
<td>57</td>
<td>BC141063</td>
</tr>
<tr>
<td>Dmnt3a</td>
<td>CTGGTGTGGAAGACTGGGCG</td>
<td>318</td>
<td>57</td>
<td>BC172199</td>
</tr>
<tr>
<td>Dmnt3b</td>
<td>GACAGGGCAGAAATCAATACAG</td>
<td>532</td>
<td>57</td>
<td>NM_181813</td>
</tr>
<tr>
<td>Hmt1</td>
<td>CCCAGGCTGCGTCACCAAACTG</td>
<td>320</td>
<td>57</td>
<td>BC109796</td>
</tr>
<tr>
<td>Ilf3</td>
<td>TATCTGGAGAAGAATCGACATG</td>
<td>301</td>
<td>59</td>
<td>XM_592855</td>
</tr>
<tr>
<td>Telomere</td>
<td>GGCTTTGCTTTGGGGGTTTTTGGG</td>
<td>79</td>
<td>56</td>
<td>NT_039202</td>
</tr>
</tbody>
</table>

Dmnt, DNA methyltransferase; Hmt1, hnRNP methyltransferase-like 2; Ilf, interleukin enhancer binding factor.
The quantification of all mRNA transcripts was carried out by real-time qRT-PCR. Five replicate PCR experiments were conducted for all genes of interest. Experiments were conducted to contrast relative levels of each transcript and histone H2a.z in every sample. PCR was performed by adding a 4 μl aliquot of each sample to the PCR mix containing the specific primers to amplify H2a.z, DNA methyltransferase (Dnmt) 1, Dnmt3a, Dnmt3b, hnRNP methyltransferase-like 2 (Hmt1), and interleukin enhancer binding factor 3 (Ilf3). Primer sequences, annealing temperature, and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. For quantification, real-time PCR was performed as described above. The comparative cycle threshold (CT) method was used to quantify expression levels (12). Quantification was normalized to the endogenous control, H2a. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔΔCT value was determined by subtracting the H2a.z CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula 2^-ΔΔCT.

Statistical analysis. Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. Cleavage and embryo development was analyzed using one-way repeated-measures ANOVA with arcsine transformation. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using Student-Newman-Keuls method) was used for the analysis of mtDNA, percentage of methylation, and differences in mRNA expression assayed by quantitative RT-PCR. The mean of the male and female telomere length were compared using an independent samples t-test.

RESULTS

In vitro embryo development and sex ratio of bovine embryos. Sexing was performed on zona-free embryos with a single PCR using the male-specific primer, Bry4a, and a satellite Sat1. The proportion of female and male blastocysts obtained with X- and Y-chromosome-bearing sperm in the preliminary study was 87.2 and 80.3%, respectively (Fig. 1A, Table 3). The proportion of zygotes cleaving at 48 hpi was not different between X- and Y-sorted groups (58.2 vs. 55.1%, respectively); however, both groups were significantly different from the unsorted group (86.0%, P < 0.001). Furthermore, the proportion of blastocysts formed on day 7 and 8 followed the same trend; there was no significant difference between X- and Y-sorted groups (day 7: 10.1% vs. 13.5%, day 8: 13.8% vs. 18.3%, respectively) but significantly more blastocysts were produced in the unsorted group (day 7: 51.6%, day 8: 55.4%, P < 0.001; Table 4).

mtDNA copy number and telomere length in male and female blastocysts. For mtDNA quantification we used single PCR using the male-specific primer, Bry4a, and a satellite Sat1. The proportion of female and male blastocysts obtained was 87.2% and 80.3% (Fig. 1A). Sexing was performed on zonafree embryos with a single PCR using the male-specific primer, Bry4a, and a satellite Sat1. The proportion of female and male blastocysts obtained with X- and Y-chromosome-bearing sperm in the preliminary study was 87.2 and 80.3%, respectively (Fig. 1A). The proportion of zygotes cleaving at 48 hpi was not different between X- and Y-sorted groups (58.2 vs. 55.1%, respectively); however, both groups were significantly different from the unsorted group (86.0%, P < 0.001). Furthermore, the proportion of blastocysts formed on day 7 and 8 followed the same trend; there was no significant difference between X- and Y-sorted groups (day 7: 10.1% vs. 13.5%, day 8: 13.8% vs. 18.3%, respectively) but significantly more blastocysts were produced in the unsorted group (day 7: 51.6%, day 8: 55.4%, P < 0.001; Table 4).

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found when we used pooled embryos; however, in both cases the differences between sexes were significant. The mean mtDNA copy number from the pool analysis of male and female bovine blastocysts is shown in Fig. 1B. There was a difference between genders (P < 0.05); mtDNA content average in male blastocysts was 410,000 ± 23,000 and in females was 360,000 ± 21,000. When individual male and female blastocysts were analyzed the mtDNA content average in male blastocysts was 423,000 ± 33,000 and in females was 373,000 ± 27,000.

For telomere length quantification we used single Day 7 blastocysts. To evaluate the real-time PCR method, DNA from the tail of two mouse species, Mus musculus and M. spretus, was used (M. musculus animals have long telomeres with repeats of >20 kb, and M. spretus mice have short telomeres, similar to those in bovine, with 5–10 kb repeats). The mean ATLRs for the two species were compared and found to be statistically different. The mean ATLRs and the average standard deviation for the ATLRs for the two groups were similar when we used pooled embryos; however, in both cases the differences between sexes were significant. The mean ATLRs for the two species were compared and found to be similar to those in bovine, with 5–10 kb repeats). The mean

Table 4. Effect of using sex-sorted bovine semen in IVF on the cleavage rate and blastocyst yield in vitro

<table>
<thead>
<tr>
<th>COCs, n</th>
<th>Cleaved, n (%)</th>
<th>Total*, (%)</th>
<th>Cleaved# (%)</th>
<th>Total (%)</th>
<th>Cleaved (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control†</td>
<td>157</td>
<td>135* (86.0)</td>
<td>81/157* (51.6)</td>
<td>81/157* (60.0)</td>
<td>87/157* (55.4)</td>
</tr>
<tr>
<td>X-sorted</td>
<td>1,057</td>
<td>615* (58.2)</td>
<td>107/1,057* (10.1)</td>
<td>107/615* (17.4)</td>
<td>146/1,057* (13.8)</td>
</tr>
<tr>
<td>Y-sorted</td>
<td>1,094</td>
<td>603* (55.1)</td>
<td>148/1,094* (13.5)</td>
<td>148/603* (24.5)</td>
<td>200/1,094* (18.3)</td>
</tr>
</tbody>
</table>

a,bValues in the same column differ significantly (P < 0.05). Data from 5 experimental replicates. †On each day of IVF a small number of oocytes were inseminated with unsorted semen as a control to ensure procedures in the laboratory were optimal, hence the lower numbers. *Number of blastocysts from the total number of cumulus oocytes complexes (COCs); # number of blastocysts from the cleaved oocytes.

DISCUSSION

Bovine embryos derived from sex-sorted sperm have similar morphology and timing of development than those fertilized with unsorted sperm. However, at least in this study, sex-sorted bovine sperm have a lower fertility and lead to reduced embryo development compared with unsorted sperm when used in vitro. This is generally attributed to the deleterious effect of the sex-sorting procedure on the capacitation status and lifespan of sex-sorted sperm (34).
Quantitative variation in mtDNA has been associated with gamete quality and reproductive success. It has been reported that mitochondria and mtDNA genotype affect the developmental capacity of bovine oocytes in vitro. The mean copy number of mtDNA per blastocyst reported here is consistent with previous studies. It has been reported that the mtDNA copy number increases at the blastocyst stage in bovine embryos. The fact that male embryos have more copies of mtDNA indicates that this increase is faster in male than in female bovine embryos, or conversely, that degradation of mitochondria is higher in female than in male embryos during early development. It has been hypothesized that a divergence in energy metabolism is at the root of the differences between the sexes in mammals, and since metabolically active cells tend to contain more mitochondria than less active ones, there should be a difference in the number and/or activity of mitochondria in developing male and female mammals.

It has been reported in humans that telomeres on early male embryo Xq5s are ~1,100 bp shorter than on female Xq5s. In mice and rats it has been reported that telomere lengths are shorter in adult and new born males than in females. It has been also been reported that early mammalian embryos have a telomerase-dependent genetic program that elongates telomeres to a defined length. Analysis revealed no significant increase in telomere length between day 8 and 13.5 of mouse embryogenesis compared with length at the morula-blastocyst transition, indicating that telomere elongation during embryogenesis is restricted to the preimplantation morula-blastocyst transition. Because we have identified differences at the blastocyst stage between male and female embryos, it is possible that the differences between sexes at birth are consequences of the differences generated during the preimplantation period. We do not know the origin of these differences between sexes, but recently a locus with a major effect on telomere length on the distal X chromosome has been identified. One possibility is that this locus behaves like other described X-linked genes that are expressed at higher levels in female bovine embryos than in male embryos at the early blastocyst stage. In addition, we have found that the expression of Dnmt3a and Dnmt3b is higher in male than in female bovine embryos, in agreement with reports that these methyltransferases are regulatory regulators of telomere length.

Also, we found that some epigenetic modifications take place differentially between male and female embryos. We did not observe differences in those sequences that were hypomethylated: the surrounding genomic heterochromatic repeats regions to SarI and SarII, a region of the euchromatic repeated sequence 18S rRNA and in a region of the promoter of cytokeratin gene, nor in a part of the euchromatic SINE element art2, that was partially methylated (males 22.6 ± 7.7%, females 19.6 ± 4.5%). These findings are similar to those reported by Kang et al. (26), who found considerably hypomethylated states in SarI, 18S rRNA and the promoter of the cytokeratin gene and some degree of methylation (26%) in art2 in IVF blastocysts, except for SarII, which was reported to be methylated to some degree (27.8%) (26). This difference can be attributed to the different DNA methylation analysis method used (cloning and sequencing versus restriction enzyme analysis), as similar differences between methods have been found in others sequences such as SarI (26). However, we found differences in a unique sequence near a minisatellite repeat locus VNTR (AF012918) (26), indicating that the difference in methylation between male and female is not a genome-wide phenomenon and that there are sequence- or genomic region-specific differences in epigenetic modification between male and female bovine blastocysts. These findings present the possibility that other single-copy genes that are important for full-term development may also be differentially demethylated and may explain the differential expression of some autosomal genes.

Sex-related differences in mRNA transcription of certain genes may implicate a new epigenetic process occurring in early embryos that precedes gonadal sex commitment. DNA
methylations is vital for preimplantation embryo development, necessary for imprinting, transposon silencing, X chromosome dosage compensation, and genome stability. Methylation of the cytosines is the predominant epigenetic modification of vertebrate genome. It is catalyzed by Dnmt enzymes; Dnmt1 is the major maintenance methyltransferase, and it ensures that newly synthesized DNA retains the methylation pattern of the template; Dnmt3a and Dnmt3b are de novo methyltransferases, setting up the methyl-CG landscape of the genome early in development. We have found similar expression of Dnmt1 between male and female bovine embryos, but lower expression of Dnmt3a and Dnmt3b in females. It has been reported that DNA methylation is lower in XX ES cell lines than in XY or XO lines and that this hypomethylation is associated with reduced levels of Dnmt3a and Dnmt3b (60). They speculate that the X chromosome encodes a modifier locus whose product represses de novo methyltransferases. Cells with two active X chromosomes will overexpress the modifier and therefore have reduced levels of the enzymes (60). Also, the influence of sex chromosome constitution on the genomic imprinting of germ cells has been reported (11). They found that there is a dose-dependent demethylating effect exerted by the X chromosome (one in XY, two in XX germ cells, with both X chromosomes active). In addition, the X-coded protein ATRX is known to be involved in chromatin modification, and is dosage sensitive (14). It has also been reported that during in vitro culture in preimplantation embryos there is higher expression of genes present on the X chromosome in female than in male embryos, indicating that two X chromosome are active (19, 29, 39, 58). This could suggest that female cells will overexpress the modifier and therefore have reduced levels of the enzyme. The differences in the expression of these methyltransferase genes between male and female embryos may be necessary to establish the differences observed in gene expression between genders that take place in early postimplantation embryos (29).

Methylation of specific residues within the NH2-terminal histone tails plays a critical role in regulating eucharyotic gene expression. H3K4 is a cell cycle-regulated protein that it is cyclically phosphorylated during mitosis (52) and regulates PRMT1 activity, the type I protein-arginine methyltransferase, that is a cofactor of nuclear receptor-activated gene expression, acting in the methylation of the histone 4 arginine 3 (2). Hmt1 is the bovine homolog to human and murine PRMT1. These protein arginine N-methyltransferases have been implicated in a variety of processes, including cell proliferation, signal transduction, and protein trafficking (41). Embryos homozygous mutant for this gene failed to develop beyond embryonic day (E) 6.5 (41), and the expression of the Prmt1 gene in wild-type mice was greatest along the midline of the neural plate and in the forming head fold from E7.5 to E8.5 and in the developing central nervous system from E8.5 to E13.5 (41). The early differences in expression that we have found could be related to the surprisingly widespread sexually dimorphic gene expression in mice, as manifested by the identification of thousands of differentially expressed genes between male and female mice (59), and differential gene expression between the developing brains of male and female mice at stage 10.5 days postcoitum, before any gonadal hormone influence.

The differences in growth, metabolism, and genetic and epigenetic programming during the preimplantation stages indicate that males and females may respond differently to environmental conditions and suggest that early perturbations may have a sex-specific effect, not only during preimplantation development, but also that may lead to some subsequent effects on postnatal development (20). Undesirable postnatal sex-associated phenotypic consequences can result from the alteration of long-term genetic or epigenetic reprogramming (15) as a consequence of embryo exposure to suboptimal in vitro culture conditions. Possibly related to this, Beckwith-Wiedemann syndrome associated with hypomethylation of the KvDMR1 (DMRs: differentially methylated regions) occurs at a relatively high frequency in monozygotic twins, and in almost all cases, the affected twins are female (31, 57). It will be important to examine this relationship further and also to determine whether female bias occurs in association with other diseases with an epigenetic component. Embryos of different sex may respond differently to epigenetic alterations. By analyzing these early sex differences we will be able to exert greater control on sex ratio manipulation of domestic animals, and it will help us to understand other aspects of early embryo development, X inactivation, and epigenetic and genetic processes related to early development that may have a long-term effect on the offspring.

GRANTS

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REFERENCES


